

## CHARACTERISTICS OF INDIAN TUBERCLE BACILLI\*

BY

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As result of the studies undertaken during the last 4 years at the Tuberculosis Chemotherapy Centre, Madras, some characteristics of Indian tubercle bacilli which differ from those of British tubercle bacilli have been observed. These factors relate to their virulence in the guinea-pig, susceptibility to hydrogen peroxide, and drug sensitivity. The Indian and British cultures are, however, closely similar in the majority of their in vitro characteristics.

Before proceeding further, it must be emphasized, that the findings reported in this paper apply to cultures obtained from a rather selected group of patients. These patients presented themselves because of symptoms and the vast majority of them were positive on smear examination and had moderately or far advanced disease. Furthermore, all the patients were residents of Madras city.

### Virulence:

It had previously been suggested by Dhayagude and Shah (1948), Fridodt-Moller *et al* (1956), and Singh (1967), that a proportion of Indian cultures of tubercle bacilli were less virulent for guinea-pigs than European strains. It was possible, however, that this difference in the guinea-pig virulence of Indian tubercle bacilli could have been due to one of the following :

1. The difference in the breed of animal used in India as compared to Europe.
2. The environmental and dietary conditions under which the animals are kept in India.
3. The difference in the technique used, or
4. that the strains tested were not typical tubercle bacilli, but anonymous mycobacteria.

The problem of virulence in the guinea-pig of Indian tubercle bacilli was, therefore, re-investigated, with specific emphasis on the elucidation of the role of these possible factors. The experiments were so designed that cultures from Indian patients in Madras and British patients in England tested in 3 laboratories-2 in England and 1 in Madras in 3 breeds of guinea-pigs-1 bred in India for a long time at Madanapalle, 1 brought out from England and used both in London and Madras, and the 3rd bred at Porton, Salisbury, England. The

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cultures were flown from India to England and vice versa. All the cultures were tested by a series of *in vitro* identification tests to ensure that they were typical mammalian tubercle bacilli.

The virulence in the guinea-pig were determined from the extent of the disease present at 6 and 12 weeks after inoculating 1 mg. moist weight of bacilli intramuscularly. The extent of the disease was assessed by a scoring procedure, the possible scores ranging from 0 to 100.

The best measurement of virulence was found to be an *index* obtained by dividing the score by the survival time of the animals in days (Mitchison *et al*, 1960). It indicated the rate of development of lesions and also by inference the rate of multiplication of the bacilli in the organs of the guinea-pig. It also allowed comparison of the results, irrespective of whether death had occurred before the day appointed for autopsy.

No significant difference was found between the different experiments and there was no marked difference between the different breeds of the guinea-pigs or between the assessment carried out in different laboratories. In addition to the comparison of the post-mortem scores and the indices of virulence, 2 other methods were employed to compare the virulence. One was the mortality of guinea-pigs and the other was the frequency and degree of positivity of cultures from the spleen of guinea-pigs infected with Indian cultures, as compared with those infected with British cultures. All these 3 methods showed that Indian cultures differ from British cultures in that they have a wider range of virulence, approximately 30 per cent being as virulent as the British cultures, 40 per cent having an intermediate degree of virulence and 30 per cent being markedly attenuated.

The frequency distribution of the mean indices of virulence of the 49 Indian and 10 British pretreatment drug-sensitive cultures studied by Mitchison *et al* (*loc. cit.*) is presented in Table I. The mean index of virulence is the average of the 6-week and 12-week indices. While none, of the 10 British cultures had a mean index of virulence of less than 0.8, 34 of the 49 Indian cultures were in this group. Conversely, all 10 British cultures had a mean index of more than 0.8 as compared to only 15 of the 49 Indian cultures. The mean index of virulence for the Indian cultures was 0.64, compared with 1.12 for the British cultures. This difference between the Indian and British cultures attains statistical significance ( $P < 0.001$ ).

The practical implications of this finding are :-

First, in India reliance cannot be placed on the guinea-pig as a means of isolating tubercle bacilli diagnostically; and in particular from specimens in which the bacilli are expected to be present only in small numbers.

Secondly, the identification of Indian cultures of tubercle bacilli as *Mycobacterium tuberculosis* cannot be based on their virulence for the guinea-pig.

TABLE I

*Virulence in the guinea-pig of pretreatment Indian and British cultures of tubercle bacilli*

| Mean index of virulence | INDIAN CULTURES |    | BRITISH CULTURES |       |
|-------------------------|-----------------|----|------------------|-------|
|                         | No.             | %  | No.              | %     |
| 0.0—                    | 4               | 8  | 0                | (0)*  |
| 0.2—                    | 10              | 20 | 0                | (0)   |
| 0.4—                    | 13              | 27 | 0                | (0)   |
| 0.6—                    | 7               | 14 | 0                | (0)   |
| 0.8—                    | 10              | 20 | 2                | (20)  |
| 1.0—                    | 2               | 4  | 5                | (50)  |
| 1.2—                    | 3               | 6  | 3                | (30)  |
| Total                   | 49              | 99 | 10               | (100) |
| Mean index of virulence | 0.64            |    | 1.12             |       |

\*Brackets indicate percentages based on fewer than 25 observations

There is one rather interesting possible explanation of this finding of so many attenuated strains in India. Since there is no evidence that the Indian strains are less infective than the British strains, it is possible that these attenuated cultures exist in India, because under the conditions applying here, they have a relative biological advantage over virulent strains. In this population, with high risk of infection and with malnutrition, the attenuated strains may more often be able to cause chronic cavitating disease and thus continue to exist. Attenuation may, therefore, be a mechanism to establish a balance between the susceptibility of the host and the virulence of the parasite.

It had also been shown (Bhatia *et al*, 1961) that cultures obtained from Indian patients at different times have a consistent degree of virulence. Thus, variation in virulence from culture to culture obtained at intervals of upto 6, weeks from the same untreated Indian patient, was no greater than the natural variation in response of the guinea-pigs in the virulence test and was considerably less than the variation in virulence from patient to patient. It was, therefore, meaningful to correlate difference in virulence of tubercle bacilli obtained from Indian patients before the start of chemotherapy with the severity or type of the disease and their subsequent response to treatment with anti-tuberculous drugs. In brief, there were no important associations between pretreatment virulence and the severity of the disease on admission to treatment, as assessed by total extent of the radiographic lesions, the extent of cavitation and the degree of positivity of the sputum (Tuberculosis Chemotherapy Centre, 1960). Further findings of the relationship between the virulence of pretreatment cultures and response to chemotherapy will be presented later.

Susceptibility to hydrogen peroxide :

Indian and British cultures of tubercle bacilli have also been compared in respect of their susceptibility to hydrogen peroxide (Subbaiah *et al* 1960). The

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method briefly was to expose cultures to 0.02 per cent hydrogen peroxide for 90 minutes and then to determine by viable counting the number of bacilli that survived. The number of organisms which survived exposure to hydrogen peroxide has been expressed as a percentage of the total number of organisms that were exposed.

The susceptibility to hydrogen peroxide of 8 British and 7 Indian cultures which survived exposure to hydrogen peroxide for 90 minutes is shown in Table II. All the cultures had 2 plus, that is full catalase activity and all were sensitive to isoniazid. All the 8 British cultures were relatively resistant to hydrogen peroxide, 32 per cent or more of the organisms being viable after exposure for 90 minutes. Of the 7 Indian cultures tested; 4 were markedly susceptible, 2 per cent or less of the organisms surviving.

TABLE II

*Susceptibility hydrogen peroxide of Indian and British cultures of tubercle bacilli*

| Culture No.             | Catalase activity | Percentage of organisms resistant to hydrogen peroxide |
|-------------------------|-------------------|--|
| <i>British cultures</i> |                   |  |
| I 837                   | ++                | 98   |
| I 841                   | ++                | 126  |
| I 907                   | ++                | 48   |
| I 919                   | ++                | 37   |
| I 959                   | ++                | 54   |
| I 952                   | ++                | 66   |
| I 1129                  | ++                | 65   |
| I 1107                  | ++                | 32   |
| <i>Indian cultures</i>  |                   |  |
| 28539                   | ++                | 129  |
| 30510                   | ++                | 63   |
| 31370                   | ++                | 0.10   |
| 34024                   | ++                | 0.25   |
| 34254                   | ++                | 46   |
| 39804                   | ++                | 2.1  |
| 40038                   | ++                | 1.3  |

Isoniazid-resistant organisms with reduced catalase activity are known to be attenuated in the guinea-pig. These organisms are also more susceptible to exposure to hydrogen peroxide. It is, therefore, possible that the difference in the virulence in the guinea-pig of different Indian cultures of tubercle bacilli results from the differences in their susceptibility to hydrogen peroxide. This problem in at present being investigated.

### Drug sensitivity:

The Indian strains differ from British strains in their sensitivity to PAS and thiacetazone.

Sensitivity tests to PAS were performed by the method described in detail in an earlier report from this Centre (Tuberculosis Chemotherapy Centre, 1959).

In brief, a representative sample of the cultures was inoculated on to a series of increasing concentrations of PAS in Lowenstein-Jensen medium; After 4 weeks incubation at 37°C, the degree of growth was examined. The lowest concentration of PAS which inhibited the growth of the culture was determined using 2 different definitions of growth on the drug containing slopes.

(1) Significant growth was defined as 20 or more colonies ; this is referred to as the 20-colony minimal inhibitory concentration (MIC).

(2) Significant growth was defined as presence of 100 or more colonies ; this is referred to as the 100 colony MIC.

Table III presents a comparison of the sensitivity to PAS of pretreatment Indian and British cultures of tubercle bacilli. Considering 20-colony MIC values, the Indian strains were on the average more resistant than the British strains. Values of 4 µg/ml or more were given by 38 (47 per cent) of the 81 Indian strains and by one (2 per cent) of the 52 British strains. In addition, the range of the MIC values for Indian cultures was wider than that for British cultures. The difference between Indian and British cultures was markedly reduced, however, when the 100 colonies definition of growth was used. Minimal inhibitory concentrations of 2µg/ml or more being required by 11 (14 per cent) of the 81 Indian strains and by one (2 per cent) of the 52 British strains.

TABLE III

**Comparison of the sensitivity to PAS of Indian and British cultures of tubercle bacilli.**

| Minimal inhibitory concentration of sodium PAS (µg/ml) | NUMBER OF STRAINS |            |                  |            |                 |            |                  |            |
|--|-------------------|------------|------------------|------------|-----------------|------------|------------------|------------|
|  | 20-colony MIC     |            |                  |            | 100-colony MIC  |            |                  |            |
|  | Indian cultures   |            | British cultures |            | Indian cultures |            | British cultures |            |
|  | No.               | %          | No.              | %          | No.             | %          | No.              | %          |
| >64  | 1                 | 1          | 0                | 0          | 0               | 0          | 0                | 0          |
| 64   | 2                 | 2          | 0                | 0          | 0               | 0          | 0                | 0          |
| 16   | 4                 | 5          | 0                | 0          | 0               | 0          | 0                | 0          |
| 8  | 16                | 20         | 0                | 0          | 0               | 0          | 0                | 0          |
| 4  | 15                | 19         | 1                | 2          | 5               | 6          | 1                | 2          |
| 2  | 15                | 19         | 10               | 19         | 6               | 7          | 0                | 0          |
| 1  | 13                | 16         | 15               | 29         | 14              | 17         | 7                | 13         |
| 2-5  | 10                | 12         | 13               | 25         | 11              | 14         | 14               | 27         |
| 0.25 or less   | 5                 | 6          | 13               | 25         | 45              | 56         | 30               | 58         |
| <b>Total</b>   | <b>81</b>         | <b>100</b> | <b>52</b>        | <b>100</b> | <b>81</b>       | <b>100</b> | <b>52</b>        | <b>100</b> |

Further investigations on the PAS sensitivity of tubercle bacilli (Selkon *et al.*, 1960) have confirmed that this difference between the PAS sensitivity of Indian and British cultures is genuine. It appears that this difference is due to the fact that Indian cultures of tubercle bacilli contain a small proportion (0.02 per cent or slightly less) of PAS resistant organisms which are not present in British cultures.

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This *in vitro* difference between British and Indian cultures does not appear, however, to be of any importance *in vivo*. We have not been able to observe any relationship between the presence of this small proportion of resistant strains and any tendency for the patient to fail to respond to treatment with PAS. The exact type of *in vitro* sensitivity test against PAS for Indian cultures has not as yet been determined, but it would seem that the use of a smaller inoculum, namely, 1: 10 of the normal inoculum may be suitable.

An even more marked difference exists between Indian and British cultures, in their behaviour in the thiacetazone sensitivity tests (Thomas *et al*, 1961). Indian cultures showed considerable variation in their sensitivity to thiacetazone which plus partly due technical variation, but also in part due to differences in their sensitivity to this drug. The thiacetazone sensitivity test, therefore, cannot be used as an identification test for tubercle bacilli, as in the case in Europe.

*In vivo* characteristics :

Having mentioned the differences between Indian and British cultures, it is important to emphasise their similarity and the important fact that cultures obtained from patients' resident in Madras city with clinically diagnosed pulmonary tuberculosis and invariably infected with typical human tubercle bacilli.

We admitted 341 patients to a controlled study of 4 regimens of domiciliary chemotherapy (Tuberculosis Chemotherapy Centre, 1980). Cultures from 287 of these 341 patients were examined by series of *in vitro* identification tests. These tests were:-

1. Bacterial morphology-
2. Colonial morphology on
  - (a) Lowenstein-Jensen slopes
  - (b) 7H-10 oleic acid albumen agar plates
- 3.. Growth at 23°C.
4. Pigmentation after exposure to light
6. Catalase activity of growth on
  - (a) a drug-free Lowenstein-Jensen slope
  - (b) an a slope of Lowenstein-Jensen medium containing 50 µg/ml  
isoniazid
6. Niacin production,

Cultures from the remaining 64 patients were tested for their sensitivity to isoniazid, catalase activity and photochromogenicity. In addition, a few cultures were also, tested for guinea-pig virulence. The investigation has been described in detail by Thomas *et al* (*loc. cit.*). All the 341 patients admitted to this trial yielded cultures which on the basis of these investigations were regarded as human tubercle bacilli. Of the 341 cultures examined, 2 were anonymous mycobacteria, but neither of them were regarded as the aetiological agent of the patients' disease. In both cases, these anonymous mycobacteria were found on only 1 occasion from these;

patients and other cultures were obtained from them, which were typical tubercle bacilli. The absence in this series of cases, of any instances in which anonymous mycobacteria could be incriminated, as the aetiological agent of clinically diagnosed pulmonary tuberculosis is of particular interest because of the relative frequency with which they are encountered in other countries particularly some parts of the U.S.A.

Niacin production was tested for in 277 of the 341 Indian cultures. All cultures were niacin positive and have, therefore, been regarded as human strains. This is a little surprising in view of the fact that 1.8 per cent of the cattle and 2.8 per cent of the buffaloes in Madras City, and adjacent rural areas have been shown to be tuberculin reactors.

#### Conclusion :

In conclusion, studies on Indian-cultures of tubercle bacilli have shown :-

1. They have a wider range of virulence in the guinea-pig than British cultures, approximately 30 per cent being as virulent as British cultures.
2. A proportion of Indian cultures show greater susceptibility to hydrogen peroxide than the British cultures.
3. Indian cultures behave differently to British cultures in the standard sensitivity tests to PAS and thiacetazone.
4. The aetiological agent of clinically diagnosed pulmonary tuberculosis in patients resident in Madras city is *Mycobacterium tuberculosis* var. *hominir*.

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